Eur. J. Biochem. 66, 229–241 (1976)

Bacillus subtilis Phage Φ29

Characterization of Gene Products and Functions

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(Received February 23/April 12, 1976)

A total of 22 Φ29-induced proteins have been resolved by slab gel electrophoresis; two of these proteins are the precursor and product fragment, respectively, in the synthesis of the neck appendage protein of the phage. The protein products of 10 out of the 17 cistrons detected in the genome of phage Φ29 have been identified. Mutants in two other cistrons fail to synthesize two proteins. Mutants in six genes to not synthesize phage DNA. A cistron, probably involved in the final lysis of the infected bacteria, has been found. Mutants in this gene give place, under restrictive conditions, to delayed lysis and produce, after artificial lysis, a burst size similar or higher than that obtained after wild-type phage infection.

Bacteriophage Φ29 contains a double-stranded DNA of molecular weight 12×10^6 [1] (and A. Talavera, personal communication) closed by a protein [2].

By infection of ultraviolet-irradiated *Bacillus subtilis* with phage Φ29 we have previously shown the induction of 19 proteins [3]. Nine of these proteins are early and ten, including the seven structural proteins, appear at late times after infection. 23 and 21 proteins, respectively, have been reported by Hawley *et al.* [4] and by McGuire *et al.* [5], to be induced in *B. subtilis* after Φ29 infection. Two of these proteins, however, are the precursor and product fragment in the synthesis of the neck appendage protein [6, 7]. Thus, up to now a maximum of 21 proteins have been detected after Φ29 infection.

A total of 17 complementation groups were identified in phage Φ29 [8] by using the collection of *ts* mutants from Talavera *et al.* [9] and that of *sus* mutants from Moreno *et al.* [8]. More recently, the mutants from the collections of Reilly *et al.* [10], Talavera *et al.* [9] and Moreno *et al.* [8] have been integrated in a common genetic map with a total of 17 genes (Mellado, Moreno, Viñuela, Salas, Reilly and Anderson, submitted to *J. Virol.*). Fig. 1 shows a genetic map of phage Φ29 with the genes named according to the new nomenclature (Mellado *et al.*, submitted).

Most of the early proteins induced after Φ29 infection are phage-coded since they are synthesized in a cell-free system *in vitro* directed by Φ29 DNA [11].

Enzymes. Pancreatic ribonuclease (EC 3.1.4.22); lysozyme or mucopeptide *N*-acetylmuramylhydrolase (EC 3.2.1.17).

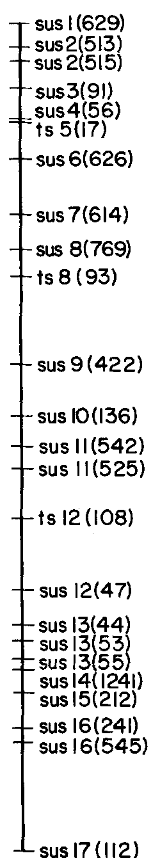

Fig. 1. *Genetic map of phage Φ29.* This map is adapted from that of Mellado *et al.* (submitted paper)

The characterization of the suppressor strain su^{+3} [12] used to isolate the suppressor-sensitive mutants of Φ29, as a suppressor of nonsense mutations [13] has

allowed the identification of the protein products of different genes [5, 6, 13, 14].

In this paper we report the assignment of ten Φ29-induced proteins to a specific cistron, among the 17 genes identified until now. For two cistrons, 1 and 8, two proteins were missing after infection with the *sus* mutant. In the case of cistron 8, different *sus* mutants have been used and always the two proteins were lacking.

We have also studied the synthesis of viral DNA after infection of *B. subtilis* with mutants in the 17 complementation groups under restrictive conditions. Previously, up to three different cistrons were reported to be involved in Φ29 DNA synthesis [15, 16, 5]. We show here the existence of six cistrons involved in phage-specific DNA synthesis, out of the 17 genes identified in Φ29.

We have identified a cistron (gene 14) probably involved in the final lysis of the infected bacteria; mutants in this gene give place, under restrictive conditions, to delayed lysis and produce, after artificial lysis, a burst-size similar or higher than that obtained after wild-type phage infection.

MATERIALS AND METHODS

Bacteria and Phage

The nonpermissive host, *B. subtilis* 110NA try$^-$ spoA$^-$ su$^-$ and the permissive bacteria, *B. subtilis* 168 MO-99 [met$^-$thr$^-$]$^+$ su^{+3} spoA$^-$ have been described [8].

The suppressor-sensitive (*sus*) mutants of phage Φ29, 2(513), 2(515), 3(91), 4(56), 9(422), 10(74), 10(136), 11(542), 12(47), 13(53), 14(1241), 15(212), 16(241), 16(545) and 17(112) were from the collection of Moreno *et al.* [8]. Mutants *sus*7(81) and *sus*8(22) were from the collection of Mellado *et al.* [17]. The *sus* mutants 1(629), 6(626), 7(614) and 8(769), were from the collection of Reilly *et al.* [10]. Mutant *sus*14(1242) was obtained by backcrossing the double mutant *ts*11(119)*sus*14(1241) and isolating the *sus* mutation.

Bacteriophage Φ29 and empty heads, labelled with [^{14}C]leucine, were prepared as described [18].

DNA Synthesis in Φ29-Infected Bacteria in the Presence of 6-(p-Hydroxyphenylazo)-uracil

B. subtilis 110NA su$^-$ was grown in a defined medium as described [3] until the cell concentration was 1×10^8/ml; the cells were concentrated 5-fold by centrifugation and resuspension in the same medium except that amino acids were 0.5 mM and 0.4 mM 6-(*p*-hydroxyphenylazo)-uracil (a gift from Dr N. C. Brown) was added to inhibit host DNA synthesis [19]. The cells were infected with Φ29 wild-type or with the different mutants at a multiplicity of 20 and labelled either with [^{14}C]uracil (0.5 µCi/ml, 4 µCi/mol) as described [15] or with [^{3}H]thymidine (2 µCi/ml; 19000 Ci/mol) in the presence of uridine (200 µg/ml) [5]. A control was kept uninfected. The cultures were shaken at 42 °C except where indicated otherwise. At different times aliquots were removed from the culture to assay for radioactivity insoluble in cold 5% trichloroacetic acid. When radioactive uracil was used the samples were treated previously with alkali as described [15]. At several times, aliquots were taken from the infected cultures to follow phage development.

Lysis of B. subtilis *110NA Infected with* sus *and* ts *Mutants of Phage Φ29*

A culture of *B. subtilis* 110NA su$^-$ grown in broth [8] at a concentration of about 10^8 cells/ml was infected with Φ29 wild-type or with different mutants at a multiplicity of 10. A control was kept uninfected. The cells were shaken at 37 °C, or in the case of mutant *ts*6(1360) at 42 °C, and at different times the absorbance at 570 nm was determined. Aliquots were also taken at several times to follow phage development.

Ultraviolet Irradiation and Labelling of Bacteria

B. subtilis 110 su$^-$ was grown in minimal medium and irradiated with ultraviolet light for 7.5 min prior to infection as described [3]. The irradiated bacteria were resuspended at a density of 5×10^8 cells/ml in minimal medium containing 0.1 mM amino acids, infected with phage Φ29 or with different *sus* mutants at a multiplicity of 20 and shaken at 37 °C. At 5 and 20 min postinfection, aliquots of the culture were labelled with 5 µCi/ml of a ^{14}C-labelled amino acid mixture (58 Ci/atom carbon, obtained from The Radiochemical Center, Amersham). 8 min later a 10-fold excess of unlabelled amino acids were added and the incubation was continued for 2 min. As a control, irradiated, uninfected bacteria were pulse-labelled at the same times in the same conditions. After the chase, the bacteria were cooled in a bath of ice-water and sedimented by centrifugation. Each pellet was resuspended in half the original volume of a buffer containing 0.01 M sodium phosphate, pH 7.2, 1 mM EDTA, 0.58 mM phenylmethylsulfonyl fluoride and lysozyme, 500 µg/ml, incubated for 2 h at 0 °C, frozen and thawed three times, treated with pancreatic RNase (10 µg/ml) for 30 min at 0 °C and prepared for gel electrophoresis as indicated below.

When double-label experiments were carried out, the mutant-infected cells were labelled with [^{14}C]-leucine (7 µCi/ml, 0.02 mM) and uninfected cells with [^{3}H]leucine (25 µCi/ml, 0.02 mM).

Polyacrylamide Gel Electrophoresis

Gel electrophoresis was carried out in 30-cm-long, 1.5-mm-thick and 14-cm-wide slabs. The separation gel was prepared by forming a linear gradient with two solutions, one containing 20% acrylamide and 0.33% *N,N'*-methylenebisacrylamide and the other containing 10% acrylamide and 0.25% *N,N'*-methylenebisacrylamide in the presence of 0.325 M Tris-HCl, pH 8.8, 0.1% sodium dodecylsulfate, 0.05% tetramethylethylenediamine and 0.0125% ammonium persulfate. The stacking gel was as described [20]. The electrophoresis buffer contained 0.025 M Tris/0.192 M glycine, pH 8.6 and 0.1% sodium dodecylsulfate. The samples for electrophoresis were prepared by heating for 2 min in a bath of boiling water in a buffer containing 0.0625 M Tris-HCl, pH 6.8, 2% sodium dodecylsulfate, 5% 2-mercaptoethanol and 6 M urea; 0.075–0.1 ml was loaded on each sample well, which was 4-mm wide. Electrophoresis was carried out at room temperature for 15 h at a constant current of 20 mA per slab. After electrophoresis the gel slabs were dried under vacuum and autoradiography was performed on Kodirex X-ray film [21]. Densitometry was carried out with a Chromoscan MK II densitometer at 610–690 nm.

In some cases, electrophoresis in tubes was carried out. The separation gels, 24-cm long, were prepared by forming a linear gradient with 10% and 20% acrylamide solutions as described above. The stacking gel and electrolyte were also as described before; 0.2 ml of sample was loaded in each gel. Electrophoresis was carried out at a constant current of 1.5 mA per gel for 14 h. The gels were cut in fractions of 1 mm with a Mickle gel slicer and counted as described [3].

RESULTS

DNA Synthesis in B. subtilis *110NA su⁻ Infected with* sus *or* ts *Mutants of Phage Φ29 in the Presence of 6-(*p*-Hydroxyphenylazo)-uracil under Restrictive Conditions*

We have previously reported that phage *Φ*29 *ts* mutants in cistrons 2, 3 and 5 do not synthesize phage DNA in bacteria treated with 6-*p*-hydroxyphenylazo-uracil [15]. The same results are obtained with the *sus* mutants 2(513) and 3(91) (for cistron 5 there is no *sus* mutant available) (Tables 1 and 2). Other mutants which did not synthesize phage DNA were *sus*1(629), *ts*6(1360), *sus*6(626) and *sus*17(112) (Tables 1 and 2). The remaining *sus* mutants representative of the different cistrons, synthesize phage DNA; in some cases, the amount of DNA synthesized was higher and in other cases lower than that produced by infection with wild-type phage (Tables 1 and 2).

Table 1. *DNA synthesis in* B. subtilis *110NA su⁻ infected with* sus *or* ts *mutants of phage Φ29*
The mutants are ordered from top to bottom according to their position in the genetic map (Fig. 1). The values obtained in the uninfected control, ranging from 2% to 14% of those in wild-type infected cells were substracted in all cases. The experiments were carried out at 42 °C. Most of the values are the average of two experiments

Mutant	DNA synthesis
	%
sus⁺ or *ts*⁺	100
*sus*1(629)	< 1
*sus*2(513)	< 1
*sus*3(91)	< 1
*sus*4(56)	124
*ts*5(17)	< 1
*ts*6(1360)	< 1
*sus*6(626)	< 1
*sus*7(614)	153
*sus*8(769)	151
*sus*9(422)	109
*sus*10(74)	90
*sus*11(542)	88
*sus*12(47)	105
*sus*13(53)	100
*sus*14(1241)	88
*sus*15(212)	70
*sus*16(545)	129
*sus*17(112)	< 1

Table 2. *Summary of some properties of the Φ29 mutants*
n.t. = not tested

Mutant	DNA synthesis	Lysis	Phage development	Protein(s) missing
*sus*1(629)	–	delayed	–	d, e
*sus*2(513)	–	very delayed [a]	–	undetected
*sus*2(515)	–	very delayed [a]	–	undetected
*sus*3(91)	–	delayed	–	p3
*sus*4(56)	+	very delayed	–	late
*ts*5(17)	–	very delayed [a]	–	n.t.
*ts*6(1360)	–	very delayed [a]	–	n.t.
*sus*6(626)	–	very delayed [a]	–	p6
*sus*7(614)	+	normal	–	p7, HP3
*sus*7(81)	+	normal	–	p7
*sus*8(769)	+	normal	–	HP1, HP3
*sus*8(22)	n.t.	normal	–	HP1, HP3
*sus*9(422)	+	normal	–	TP1
*sus*10(74)	+	normal	–	NP2
*sus*10(136)	+	normal	–	NP2
*sus*11(542)	+	normal	–	NP3
*sus*12(47)	+	normal	–	P-NP1, NP1, p12
*sus*13(53)	+	normal	–	undetected
*sus*14(1241)	+	very delayed [a]	+	p6, p17
*sus*14(1242)	+	delayed	+	undetected
*sus*15(212)	+	delayed	+	p15
*sus*16(545)	+	normal	–	n.t.
*sus*16(241)	+	normal	–	p16
*sus*17(112)	–	very delayed [a]	–	p17

[a] In these cases the lysis was very delayed or there was no lysis of the infected bacteria.

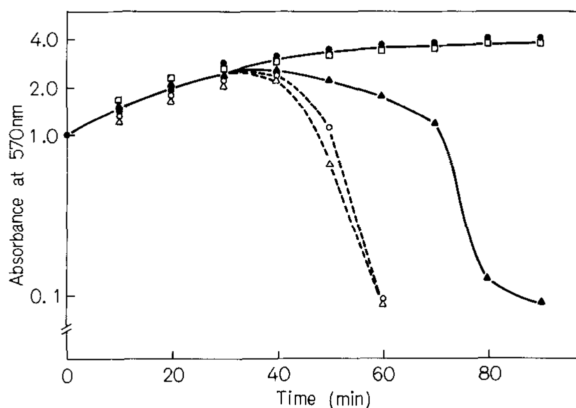

Fig. 2. *Lysis of* B. subtilis *110NA after infection with* sus *mutants of phage Φ29. B. subtilis* 110NA grown in broth up to a concentration of 10^8 cells/ml was infected at a multiplicity of 10 with wild-type phage (○----○) or with the mutants *sus*10(74) (△---△), *sus*3(91) (▲——▲) and *sus*4(56) (□——□). A control was kept uninfected (●——●). At the times indicated the absorbance at 570 nm was determined

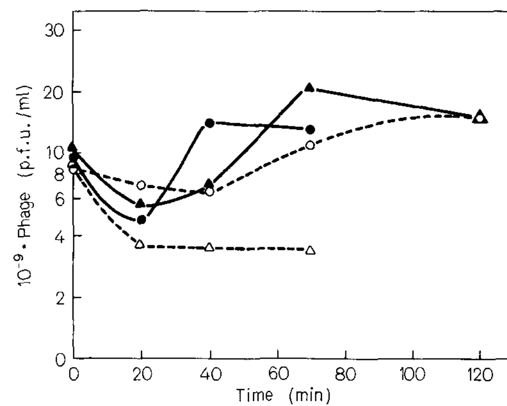

Fig. 3. *Development of Φ29* sus *mutants in ultraviolet-irradiated* B. subtilis *under restrictive conditions. B. subtilis* 110NA su⁻, grown and irradiated with ultraviolet light as indicated in Materials and Methods, was infected with phage Φ29 wild-type or with different *sus* mutants. At the times indicated, aliquots were taken to determine total phage production after lysis with lysozyme [9]. (●——●) Φ29 wild-type; (○----○) *sus*14(1241); (▲——▲) *sus*15(212); (△----△) *sus*13(53). p.f.u. = plaque-forming units

Lysis of B. subtilis *110NA su⁻ Infected with* sus *or* ts *Mutants of Phage Φ29 under Restrictive Conditions*

Fig. 2 shows the behaviour of mutants *sus*10(74), *sus*3(91) and *sus*4(56) of phage Φ29 with respect to the lysis of the infected bacteria after infection in broth, under restrictive conditions. Some of the mutants behave like *sus*10(74) and wild-type phage and produce normal lysis; these are the *sus* mutants 7(614), 8(769), 9(422), 11(542), 12(47), 13(53) and 16(545). Mutants *sus*15(212) and *sus*1(629), like *sus*3(91), produce delayed lysis. Finally, a third class of mutants behave like *sus*4(56) and do not produce the lysis of the infected bacteria or the lysis is very delayed; these mutants are *sus*2(513), *sus*6(626), *ts*6(1360), *sus*14(1241) and *sus*-17(112); mutant *ts*5(17) can be included in this third class of mutants [9] (see Table 2). The behaviour of the mutants with respect to the lysis of the bacteria is cistron-specific since in the cases where more than one mutant in the same cistron was used the same results were obtained.

Mutant *sus*14(1241) is noteworthy; as shown in Fig. 3, it gives place to normal phage production after lysis with lysozyme, although the phage development is delayed with respect to that of the wild-type phage. As will be seen later, mutant *sus*14(1241) has, in addition to the mutation in cistron 14, two other functional mutations in genes 6 and 17. When these two mutations were eliminated to obtain mutant *sus*14(1242), it behaved like mutant *sus*14(1241) except that the lysis was less delayed (Table 2). Another mutant which gave a normal phage burst under certain conditions was *sus*15(212) (Fig. 3 and Table 2). This is not the case when complementation experiments in cultures at low cell density were carried out [8]. Thus, it seems that protein p15 is dispensable under certain experimental conditions.

The rest of the mutants did not give place to phage production as is shown for mutant *sus*13(53) (Fig. 3 and Table 2).

Proteins Induced after Infection of Ultraviolet-Irradiated B. subtilis *with Phage Φ29 and with* sus *Mutants*

By electrophoresis in gels containing 12.5% acrylamide in the presence of urea and sodium dodecylsulfate, 19 phage-induced proteins were separated after infection of *B. subtilis* 110NA with phage Φ29 [3]. However, by using this system, the resolution of the proteins with molecular weights between 17000 and 10500 (proteins V to IX) was not good for the assignment of some of these proteins to specific genes. For this reason, two changes were made: (a) to use a 10–20% acrylamide gradient, in which those proteins were easily differentiated from each other; (b) to use slab gel electrophoresis instead of electrophoresis in cylindrical tubes, except in the cases in which double label was needed.

Fig. 4 A shows the scanning of the autoradiograph of a gel slab in which a lysate from Φ29-infected cells

Fig. 4. *Densitometry of the proteins induced in ultraviolet-irradiated* B. subtilis *after infection with phage Φ29.* (A) Densitometry of the slab gel electrophoresis of lysates from Φ29-infected *B. subtilis*, labelled in a 5–13-min pulse as indicated in Materials and Methods. (B) Proteins labelled in a 20–28-min pulse. (——) Φ29-infected cells; (---) uninfected cells. (C) Autoradiograph and densitometry of the slab gel electrophoresis of phage Φ29 (——) or empty heads (----) labelled with [^{14}C]leucine

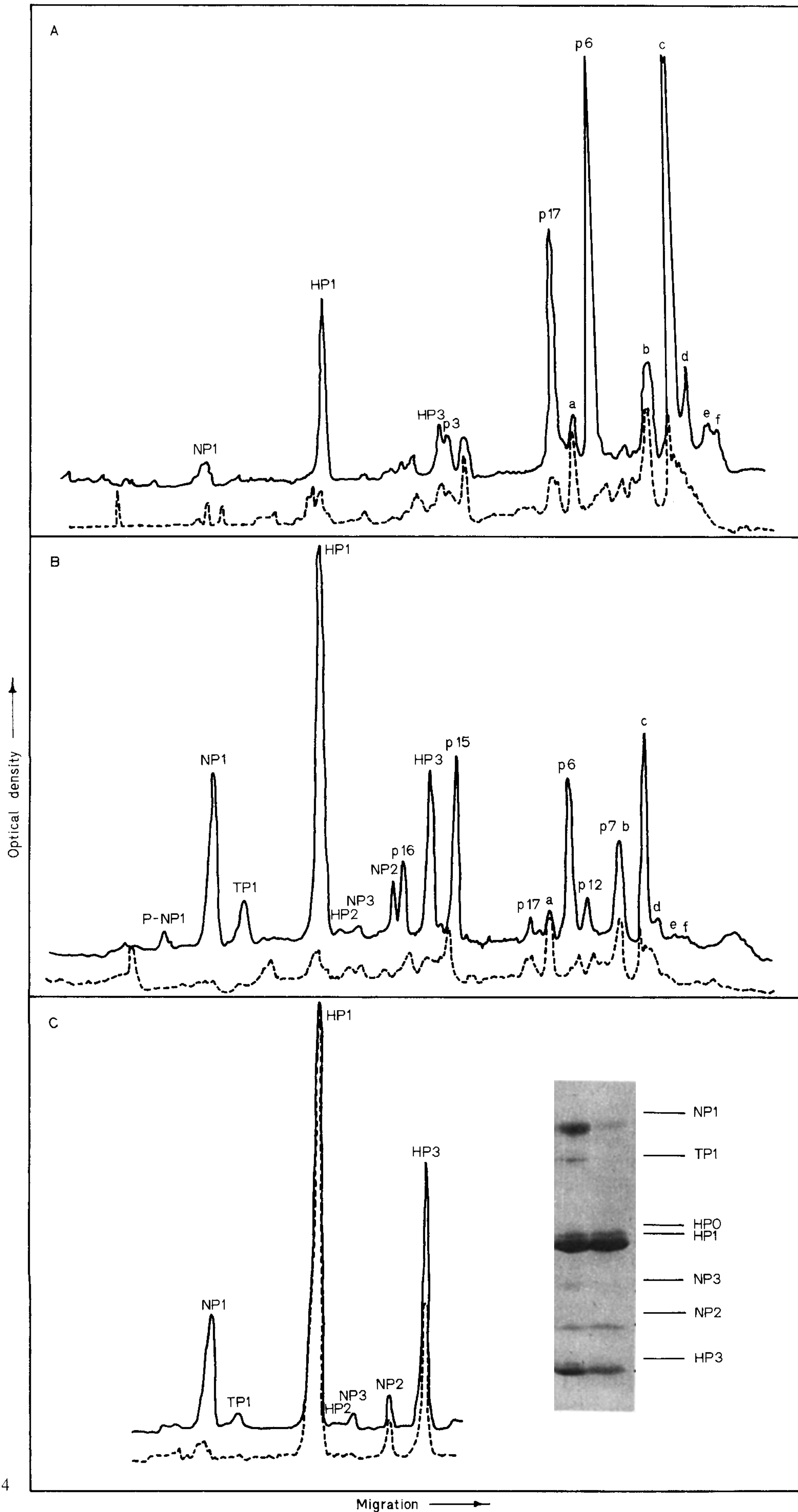

Fig. 4

labelled from 5 to 13 min postinfection (early pulse) was run. As a control the scanning of the electrophoretic bands from a lysate from uninfected cells labelled at the same time is shown. As can be seen, the nine early-induced proteins previously reported are resolved in the gel. We have named the proteins according to the gene coding for each of them (see later); thus, protein I from Carrascosa *et al.* [3], product of cistron 3, becomes p3. For six proteins we have not yet characterized the corresponding gene and they are named a to f in the order of increasing electrophoretic mobility. The synthesis of some major late proteins, like NP1, HP1 and HP3, is already apparent in this pulse. Fig. 4B shows the scanning of the autoradiograph of the proteins resolved in the gel slab from a lysate labelled in a pulse from 20 min to 28 min after infection (late pulse); a control of uninfected cells labelled at the same time is also shown. The phage structural proteins NP1 (appendages of the neck), TP1 (tail protein), HP1 (major head protein), HP2 (minor head protein), NP3 (lower collar), NP2 (upper collar), and HP3 (fibers of the head), are seen. The proteins present in complete phage and empty heads (containing the head proteins and upper collar) are shown as markers of the protein bands (Fig. 4C). As has been shown previously [22], the mobility of proteins NP2 and NP3 is inversed in the discontinuous pH system of electrophoresis with respect to that in the continuous pH system. Besides the seven proteins previously reported to be present in complete phage particles, a new protein, moving slightly slower than protein HP1, is resolved in this gel system, as can be seen in the autoradiograph from phage and empty heads, although it cannot be appreciated in the scanning of the autoradiograph (Fig. 4C). Since this protein is present in heads we will call it HP0. This protein is not seen either in the scanning of the autoradiograph of the slab gels shown in Fig. 4A and B, corresponding to the electrophoresis of lysates from infected cells labelled early and late after infection, respectively, but it can be seen in the autoradiographs of the slab gels of lysates from infected cells (see later).

Besides the phage structural proteins, the lysate labelled late after infection (Fig. 4B) shows the presence of a small amount of protein P-NP1 (the precursor of NP1), protein p12 which is probably the fragment produced by the cleavage of P-NP1 to NP1 (see later), and three other late proteins, p16, p15 and p7, as well as a reduced amount of the early proteins.

Thus, including HP0, 20 Φ29-induced proteins are resolved in this gel system besides proteins P-NP1 and the p12 fragment.

Fig. 5 shows the autoradiograph obtained after electrophoresis of the proteins labelled from 5 to 13 min, induced in ultraviolet-irradiated *B. subtilis* 110NA su⁻ infected with the *sus* mutants representative of the cistrons located in the left side of the genetic map (1 to 6). As a control, the proteins labelled in uninfected cells and those labelled in wild-type infected cells are shown. Infection with mutant *sus*1(629) does not induce the synthesis of the low-molecular-weight proteins d and e; the synthesis of late proteins in this experiment is also seen in this early pulse, due to the fact that the multiplicity of infection used was higher than 20, producing an earlier start in the synthesis of late proteins. All the early phage-induced proteins are present after infection with mutant *sus*2(515); infection with another mutant in this cistron, *sus*2(513), mapping in the middle of the gene, also produced a normal pattern of induced proteins (results not shown). Cells infected with mutant *sus*3(91) lack protein p3 (protein I from Carrascosa *et al.* [3]; molecular weight 27000). Cells infected with mutant *sus*4(56) contain all the early proteins detected so far and those infected with mutant *sus*6(626) lack protein p6. A protein band in a position

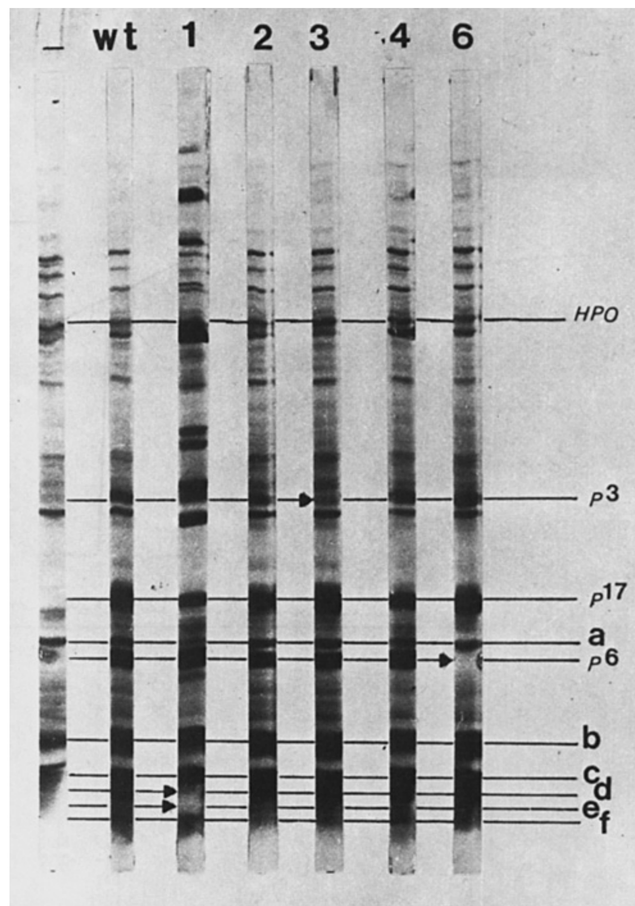

Fig. 5. *Autoradiograph of the proteins separated by slab gel electrophoresis, labelled in a 5–13-min pulse, in* B. subtilis su⁻ *infected with* sus *mutants in cistrons 1, 2, 3, 4 and 6.* The proteins labelled with ^{14}C-labelled amino acids were subjected to slab gel electrophoresis as described in Materials and Methods. The nine early proteins and protein HP0 are indicated. The arrows indicate the position where a protein band is missing. (1) Infection with mutant *sus*1(629); (2) *sus*2(515); (3) *sus*3(91); (4) *sus*4(56); (6) *sus*6(626); (–) uninfected cells; (wt) wild-type infected cells

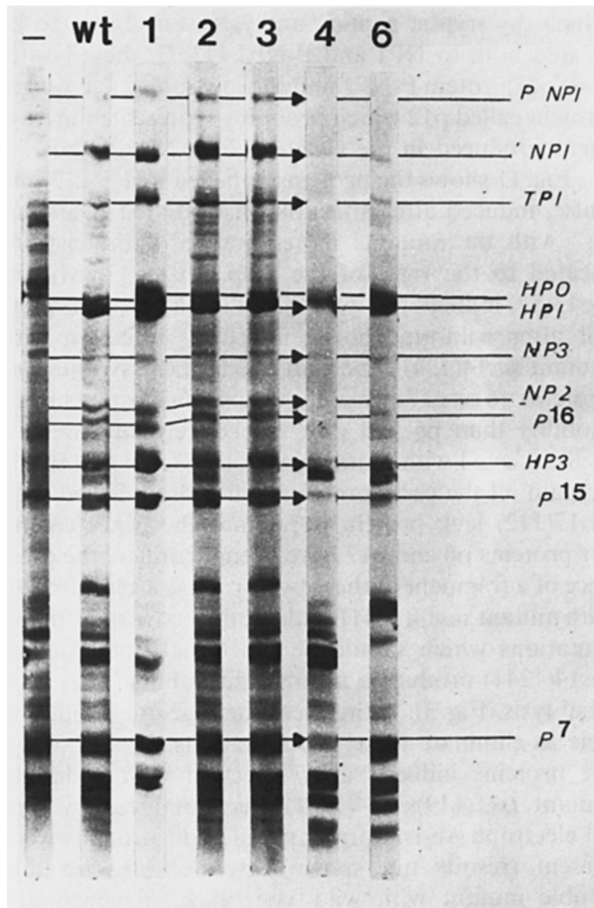

Fig. 6. *Autoradiograph of the proteins separated by slab gel electrophoresis, labelled in a 20–28-min pulse, in* B. subtilis *su⁻ infected with* sus *mutants in cistrons 1, 2, 3, 4 and 6.* The proteins labelled with ^{14}C-labelled amino acids were subjected to slab gel electrophoresis as described in Materials and Methods. The late proteins are indicated as well as protein HP0. The arrows indicate the position where a protein band is missing. (1) Infection with mutant *sus*1(629); (2) *sus*2(515); (3) *sus*3(91); (4) *sus*4(56); (6) *sus*6(626); (–) uninfected cells; (wt) wild-type infected cells

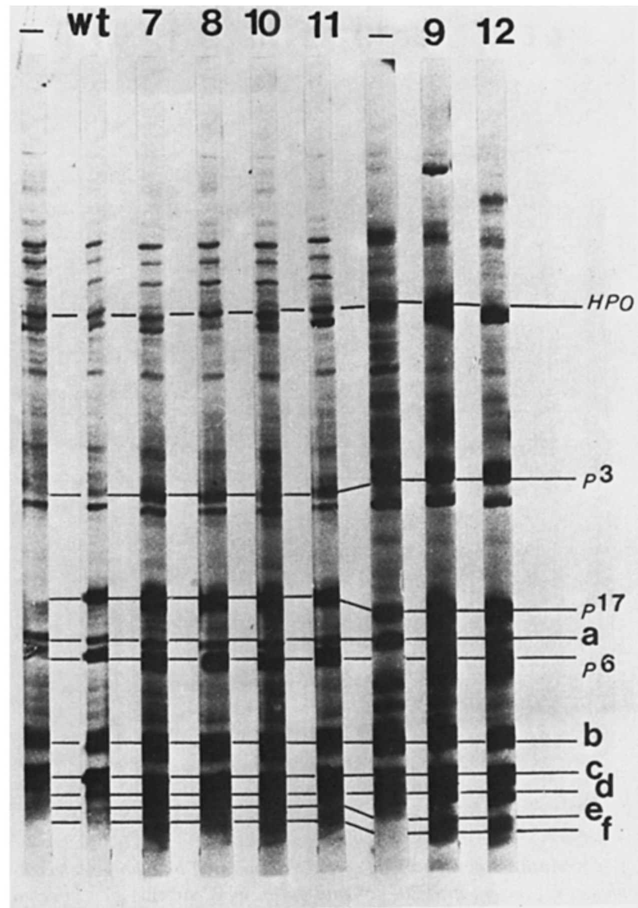

Fig. 7. *Autoradiograph of the proteins separated by slab gel electrophoresis, in* B. subtilis *su⁻ infected with* sus *mutants in cistrons 7, 8, 9, 10, 11 and 12.* The proteins labelled with ^{14}C-labelled amino acids were subjected to slab gel electrophoresis as described in Materials and Methods. The nine early proteins and protein HP0 are indicated. (7) Infection with mutant *sus*7(614); (8) *sus*8(769); (9) *sus*9(422); (10) *sus*10(136); (11) *sus*11(542); (12) *sus*12(47); (–) uninfected cells; (wt) wild-type infected cells

corresponding to that of protein HP0 is also seen; although the mobility of this protein could correspond to that of a protein present in uninfected cells, it has been assigned as a phage-induced protein since it is present in phage particles and heads (Fig. 4C).

When cells infected with the same mutants are labelled from 20 to 28 min, all late proteins are induced except in the case of the cells infected with mutant *sus*4(56) which lack all of them (Fig. 6). A protein band in a position which could correspond to that of protein HP0 is seen in *sus*4(56)-infected cells suggesting that this protein might be an early one.

Fig. 7 shows the autoradiograph of the gel pattern of the proteins labelled in a 5–13-min pulse after infection with mutants in the cistrons located in the middle region of the genetic map, 7 to 12. All of them induce the synthesis of the early proteins.

When the proteins induced after infection with these mutants are labelled from 20 min to 28 min and analyzed by slab gel electrophoresis (Fig. 8), the following results were obtained: infection with mutant *sus*7(614) does not induce the synthesis of protein p7 (protein X from Carrascosa *et al.* [3], which is a late protein of molecular weight 8000); the slightly higher mobility of protein HP3 compared with that of the normal phage protein is due to a second mutation, as indicated from the fact that other *sus* mutant in gene 7, *sus*7(81), has a normal HP3 protein; the HP3 mutation in mutant *sus*7(614) should be functional since it complements with other mutants lacking protein HP3 (unpublished results). In agreement with the results of Anderson and Reilly [6], cells infected with mutant *sus*8(769) lack the major head protein HP1 and the fiber protein HP3. Mutant *sus*10(136) does not induce the synthesis of protein NP2 and mutant *sus*11(542) seems

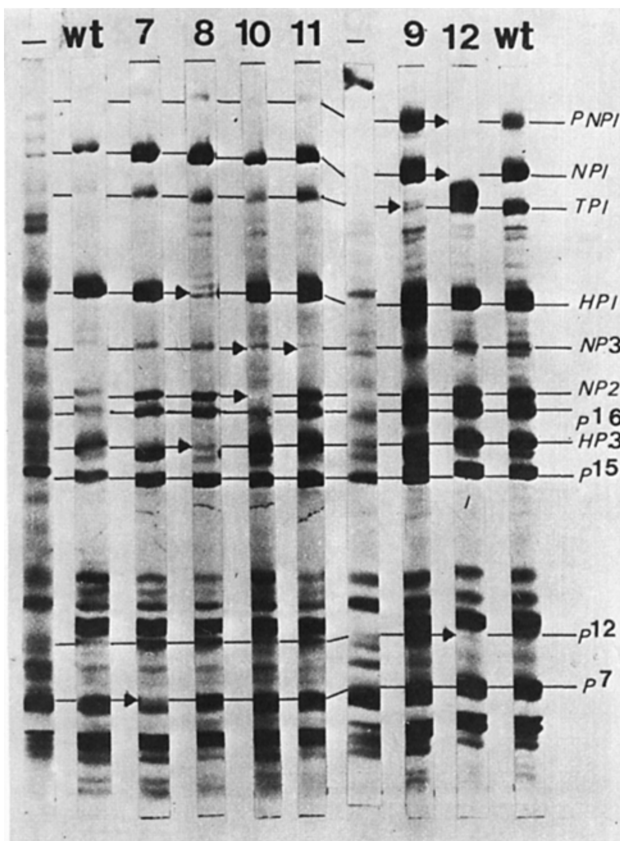

Fig. 8. *Autoradiograph of the proteins separated by slab gel electrophoresis, labelled in a 20–28-min pulse, in* B. subtilis *su⁻ infected with* sus *mutants in cistrons 7, 8, 9, 10, 11 and 12.* The proteins labelled with ^{14}C-labelled amino acids were subjected to slab gel electrophoresis as described in Materials and Methods. The late proteins are indicated. The arrows indicate the position where a protein band is missing. (7) Infection with mutant *sus*7(614); (8) *sus*8(769); (9) *sus*9(422); (10) *sus*10(136); (11) *sus*11(542); (12) *sus*12(47); (–) uninfected cells; (wt) wild-type infected cells

to lack protein NP3. As this protein overlaps with a protein present in uninfected cells, gel electrophoresis using double label was carried out of lysates from *sus*-11(542) and *sus*10(136)-infected cells labelled in a 20–28-min pulse. As shown in Fig. 9, mutant *sus*-11(542) does not induce the synthesis of protein NP3; this is clearly seen when the phage-specific radioactivity is calculated according to the method of Mayol and Sinsheimer [23]. The rest of the late proteins are induced after infection with this mutant (Fig. 8 and 9). Mutant *sus*10(136), besides lacking protein NP2 (Fig. 8), induces the synthesis of very small amounts of protein NP3 (Fig. 10), in agreement with the low complementation values obtained when *sus* mutants in cistron 10 are complemented with mutants in cistron 11, which was interpreted to be due to a polar effect [8].

Infection with mutant *sus*9(422) does not induce the synthesis of the tail protein TP1 (Fig. 8). In agreement with previously reported results, cells infected with mutant *sus*12(47) lack protein NP1, having instead a fragment of lower molecular weight ($\approx$ 75000) which, by tryptic peptide analysis, was shown to be related both to NP1 and P-NP1 [13, 7]; these lysates also lack protein P-NP1 and the low-molecular-weight protein called p12 which probably represents the fragment produced in the cleavage of P-NP1 to NP1.

Fig. 11 shows the proteins, labelled in a 5–13-min pulse, induced after infection of irradiated *B. subtilis* su⁻ with the mutants representative of the cistrons located to the right of the map, 13 to 17. Mutant *sus*13(53) induces the synthesis of all the proteins present after wild-type phage infection. Infection with mutant *sus*14(1241) does not produce the synthesis of proteins p6 or p17; instead, two proteins with a higher mobility than p6 and p17, respectively, are present. Cells infected with mutants *sus*15(212) and *sus*16(241) contain all the early proteins and those infected with *sus*17(112) lack protein p17. Since the genes coding for proteins p6 and p17 have been identified, the presence of a fragment of these two proteins after infection with mutant *sus*14(1241) could be due to two additional mutations which should be functional since mutant *sus*14(1241) produce a normal phage burst after artificial lysis (Fig. 3). If this were the case one should be able to eliminate these two mutations. In fact, when the proteins induced after infection with a double mutant *ts*11(119)*sus*14(1241) were analyzed by slab gel electrophoresis, normal p6 and p17 proteins were present (results not shown). By backcrossing this double mutant with wild-type phage, a new *sus*14 mutant, named *sus*14(1242), which induced the synthesis of normal p6 and p17 proteins, was obtained (Fig. 11). No other early protein detected so far was missing from cells infected either with mutant *sus*-14(1241) or *sus*14(1242).

Infection of *B. subtilis* with mutant *sus*14(1242), as with *sus*14(1241), also gave place to normal phage production and had a delayed lysis phenotype. The lysis delay after infection with mutant *sus*14(1242) was shorter than with mutant *sus*14(1241) (not shown), probably due to the existence of normal p6 and p17 proteins whose absence also produce delayed lysis of the bacteria.

When cells infected with mutants in cistrons 13 to 17 are labelled from 20 to 28 min, all late proteins are present after infection with mutants *sus*13(53), *sus*-14(1241) and *sus*17(112) (Fig. 12). Since the protein synthesis after infection with mutant *sus*14(1241) seems to be delayed, a pulse from 35 to 43 min was also given to try to detected all the late proteins; as can be seen, mutant *sus*14(1241) induces the synthesis of all late proteins. Proteins p15 (protein II from Carrascosa *et al.* [3]) and p16 are lacking from cells infected with mutants *sus*15(212) and *sus*16(241), respectively (Fig. 12).

Table 2 shows a summary of the proteins missing after infection of ultraviolet-irradiated *B. subtilis* with the different *sus* mutants.

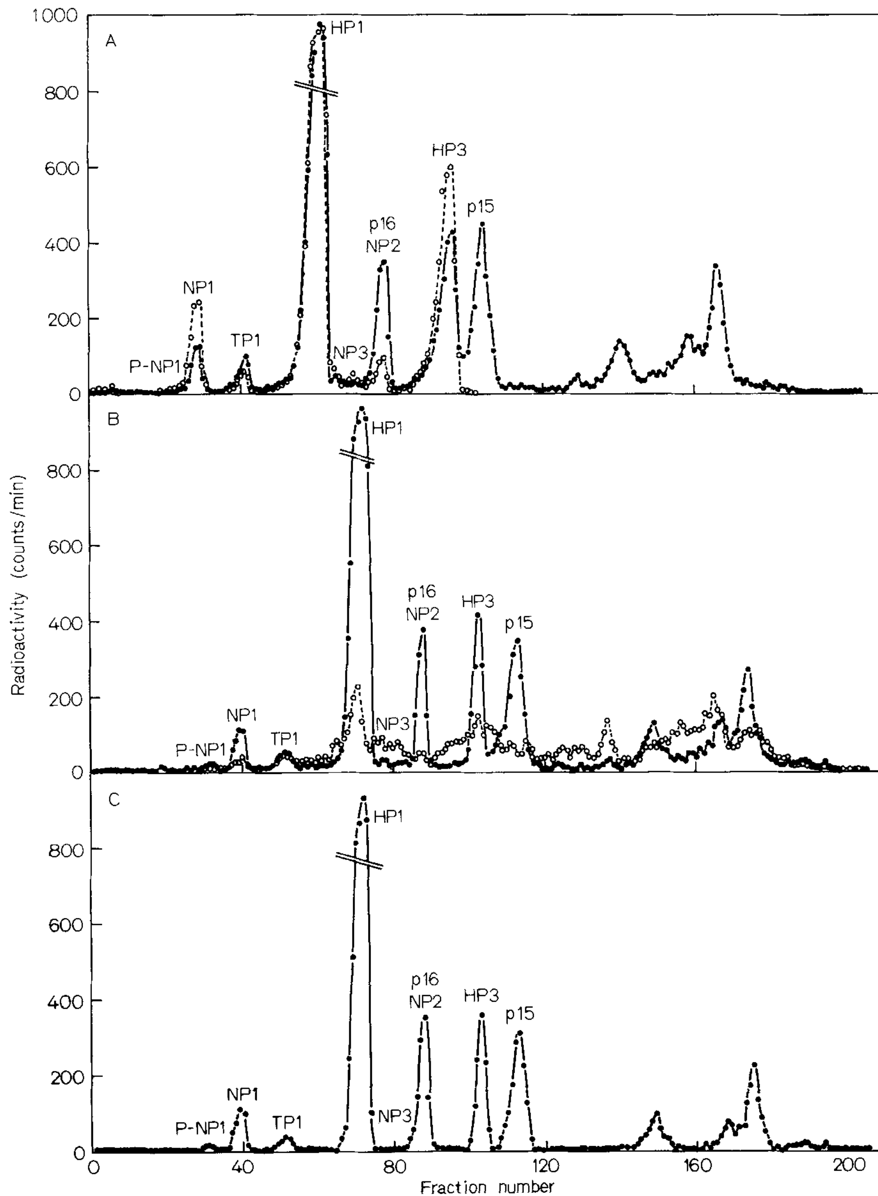

Fig. 9. *Gel electrophoresis of the proteins labelled in a 20–28-min pulse in ultraviolet-irradiated* B. subtilis *su⁻ infected with mutant* sus*11(542)*. The proteins labelled with [^{14}C]leucine in a 20–28-min pulse after infection with mutant *sus*11(542) were mixed with phage *Φ*29 labelled with [^{3}H]leucine (A) or with the proteins labelled with [^{3}H]leucine in uninfected cells at the same time (B) and subjected to tube electrophoresis as indicated in Materials and Methods; (●——●) *sus*11(542)-infected cells; (○----○) phage *Φ*29 (A) or uninfected cells (B); (C) *sus*11(542)-specific radioactivity calculated from (B) according to the method of Mayol and Sinsheimer [23]

DISCUSSIONS

Cistrons 1, 2, 3, 5, 6 and 17 are involved in phage-specific DNA synthesis (Fig. 13). One of these cistrons is probably responsible for the synthesis of a protein shown by McGuire *et al.* [5] to be involved in the attachment of viral DNA to the most membrane. Hirokawa has shown that protein associated with *Φ*29 DNA is required for transfection since transfectivity is sensitive to proteolytic enzymes [24]. More recently, Yanofsky *et al.* have reported that a *ts* mutant in cistron 3 gives place to thermolabile transfecting DNA suggesting a role for protein p3 at some stage in the process of transfection [25]. The functions for the protein products of the rest of the cistrons is presently unknown.

It is remarkable that in the small genome of phage *Φ*29, at least 6 out of the 17 genes identified are involved in DNA synthesis. The *Φ*29-specific DNA synthesis seems to be independent from the host DNA polymerase III, since it has been shown to take place in the presence of the drug 6-(*p*-hydroxyphenylazo)-uracil [15, 16, 5], a specific inhibitor of this enzyme [26, 27]. Thus, it is possible that the DNA synthesis

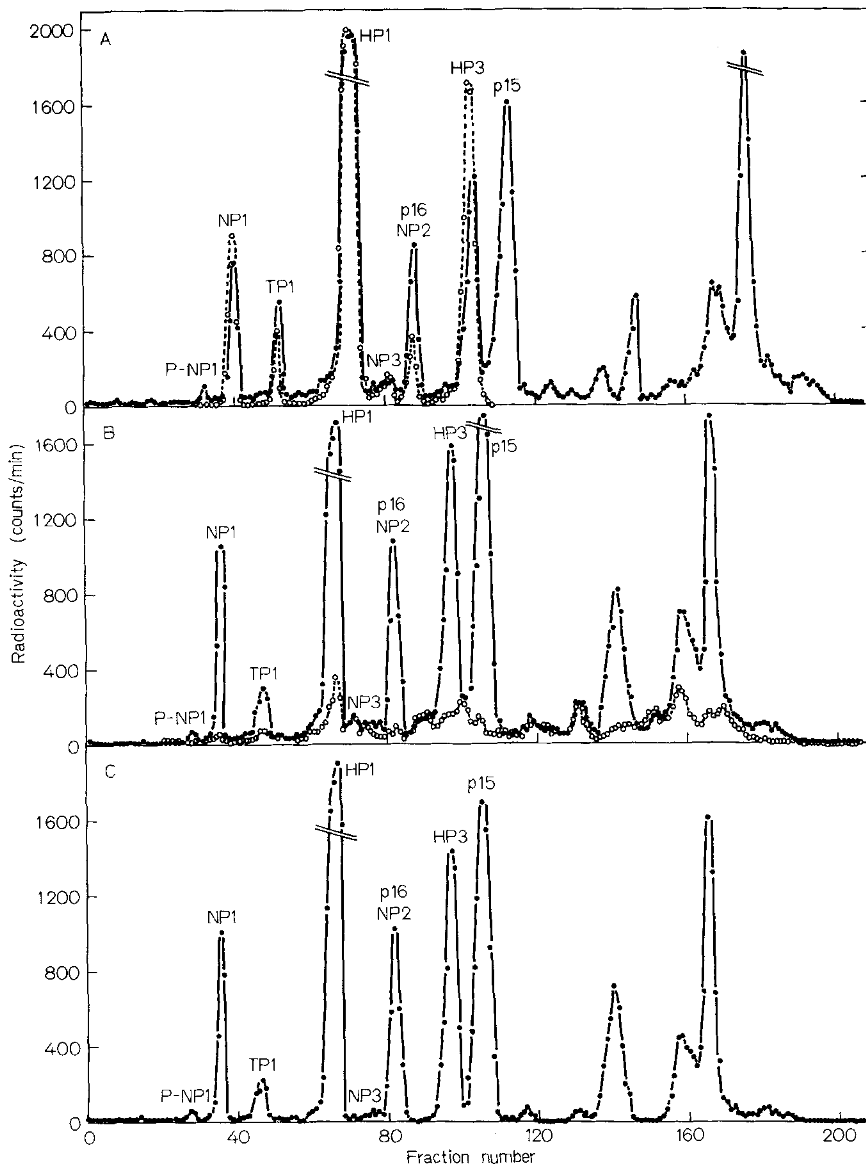

Fig. 10. *Gel electrophoresis of the proteins labelled in a 20–28-min pulse in ultraviolet-irradiated* B. subtilis *su⁻ infected with mutant* sus*10(136)*. The proteins labelled with [^{14}C]leucine in a 20–28-min pulse after infection with mutant *sus*10(136) were mixed with phage Φ29 labelled with [^{3}H]leucine (A) or with the proteins labelled with [^{3}H]leucine in uninfected cells at the same time (B) and subjected to tube electrophoresis as indicated in Materials and Methods; (●——●) *sus*10(136)-infected cells; (○- - -○) phage Φ29 (A) or uninfected cells (B); (C) *sus*10(136)-specific radioactivity calculated from (B) according to the method of Mayol and Sinsheimer [23]

ability of phage Φ29 is reasonably independent from the host DNA synthesis machinery.

Mutants in cistron 4 do not induce the synthesis of any of the late proteins. All early proteins detected so far are present in these mutants and therefore we do not know at present which is the protein affected by the mutation. Hybridization-competition experiments with the RNA synthesized in *B. subtilis* infected with mutant *sus*4(56) have shown that no late RNA is produced, indicating that the primary control of the protein product of this gene is at the level of transcription (unpublished results).

Experiments are presently being carried out to get a better resolution of the phage-induced proteins by using two-dimensional electrophoresis [28], with the aim of identifying the protein products of cistron 4 and of some other cistrons whose protein products have not yet been identified.

Cistron 14 is interesting and useful in the sense that it produces normal phage burst after artificial lysis and has a delayed lysis phenotype. It is being used in studies on the morphogenesis of the phage particle by construction of double mutants of the cistron to be studied and the *sus*14(1242) mutant.

Mutants in cistron 15 also produce, under certain infection conditions, a normal phage burst. It codes for a late, non-structural protein, present in the particles produced after infection with mutants in several cis-

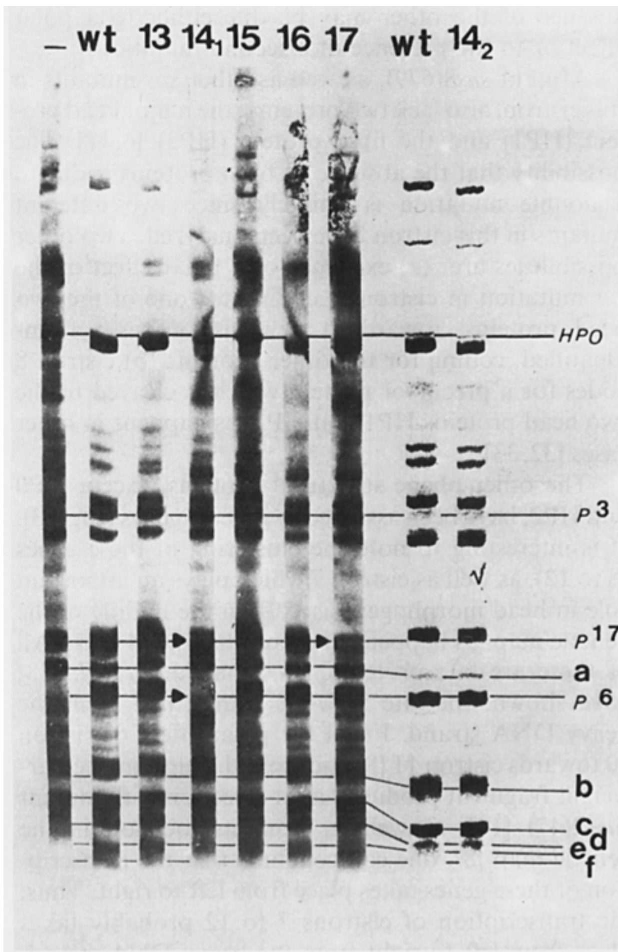

Fig. 11. *Autoradiograph of the proteins separated by slab gel electrophoresis labelled in a 5 – 13-min pulse in* B. subtilis *su⁻ infected with* sus *mutants in cistrons 13, 14, 15, 16 and 17.* The proteins labelled with ^{14}C-labelled amino acids were subjected to slab gel electrophoresis as described in Materials and Methods. The nine early proteins and protein HP0 are indicated. The arrows indicate the position where a protein band is missing. (13) Infection with mutant *sus*13(53); (14_1) *sus*14(1241); (14_2) *sus*14(1242); (15) *sus*15(212); (16) *sus*16(241); (17) *sus*17(112); (–) uninfected cells; (wt) wild-type infected cells

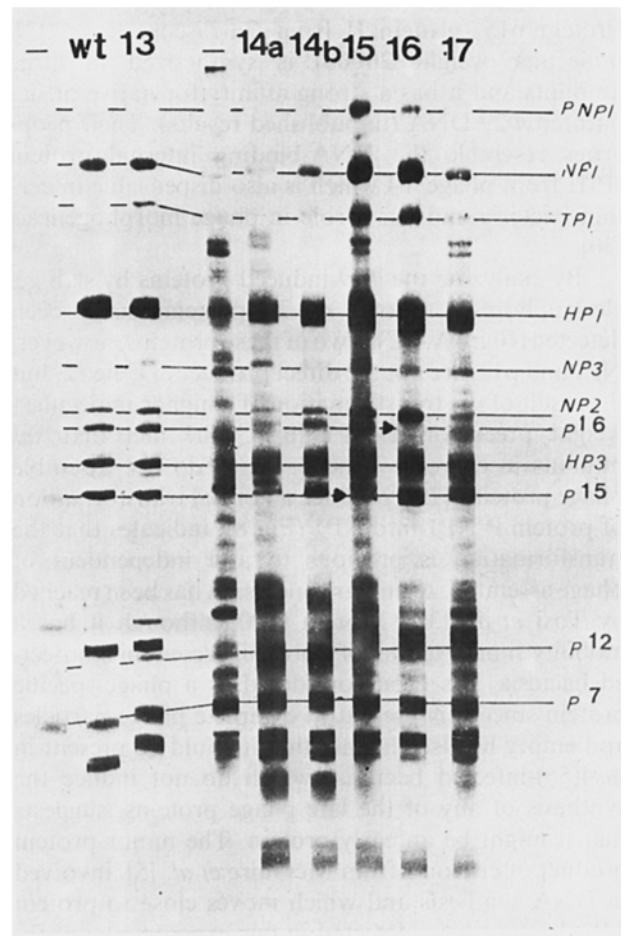

Fig. 12. *Autoradiograph of the proteins separated by slab gel electrophoresis labelled in a 20 – 28-min pulse in* B. subtilis *su⁻ infected with* sus *mutants in cistrons 13, 14, 15, 16 and 17.* The proteins labelled with ^{14}C-labelled amino acids were subjected to slab gel electrophoresis as described in Materials and Methods. The late proteins are indicated. The arrows indicate the position where a protein band is missing. (13) Infection with mutant *sus*13(53); (14a) *sus*14(1241) (20 – 28-min pulse); (14b) *sus*14(1241) (25 – 43-min pulse); (15) *sus*15(212); (16) *sus*16(241); (17) *sus*17(112); (–) uninfected cells; (wt) wild-type infected cells

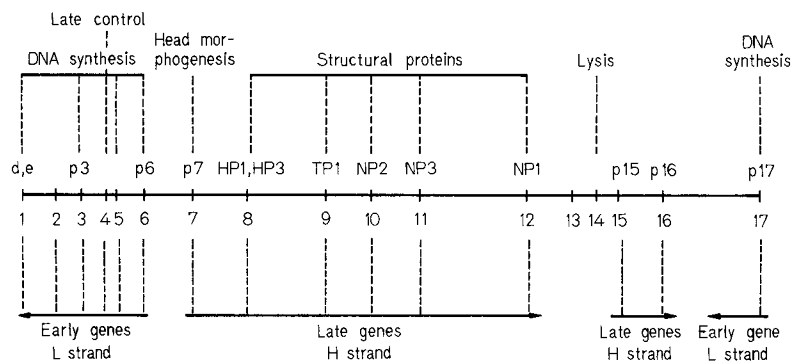

Fig. 13. *Gene linkage and direction of transcription in phage Φ29 DNA.* The numbers indicate genes. Genetic distances have been drawn proportional to recombination frequencies. A p before a gene number refers to the identified polypeptide chain of that gene. HP1, HP3, TP1, NP2, NP3 and NP1 refer to the phage structural proteins. d and e refer to the polypeptides missing after infection with mutant *sus*1(629). The arrows indicate direction of transcription

trons, suggesting a role in phage morphogenesis [29]. Protein p15 (protein II from Carrascosa *et al.* [3], molecular weight 26000) is synthesized in large amounts and it has a strong affinity for native or denatured Φ29 DNA (unpublished results). Their properties resemble the DNA-binding internal protein IPIII from phage T4 which is also dispensable in certain bacteria and has a role in phage morphogenesis [30].

By analyzing the Φ29-induced proteins by slab gel electrophoresis a total of 22 proteins have been detected (Fig. 4A – C). Two of these proteins, however, NP1 and p12, are not the direct product of gene 12, but the result of the transformation of a higher-molecular-weight precursor, P-NP1 [6, 7]. The fact that *sus* mutants in cistrons 7 and 8, which do not assemble phage proteins [29], produce a normal transformation of protein P-NP1 into NP1 (Fig. 8), indicates that the transformation is previous to and independent of phage assembly. A similar conclusion has been reached by Tosi *et al.* [31]. Protein HP0, although it has a mobility similar to that of a protein present in uninfected bacteria, has been considered as a phage-specific protein since it is present in complete phage particles and empty heads. The fact that it could be present in *sus*4(56)-infected bacteria, which do not induce the synthesis of any of the late phage proteins, suggests that it might be an early protein. The minor protein product of cistron I from McGuire *et al.* [5], involved in DNA synthesis and which moves close to protein TP1, has not been detected in our experiments, probably due to the fact that its mobility coincides with that of a protein present in uninfected bacteria. Thus, we have identified until now a total of 20 phage-induced proteins and 17 cistrons (Mellado *et al.* submitted).

By using Φ29 nonsense mutants, the protein products of cistrons 3 (p3), 6 (p6), 7 (p7), 9 (tail protein, TP1), 10 (upper collar protein, NP2), 11 (lower collar protein, NP3), 12 (neck appendages precursor, P-NP1), 15 (p15), 16 (p16) and 17 (p17) have been identified; that is to say, 10 out of 17 cistrons. Similar results have been obtained by Anderson, Reilly and coworkers [6, 14] (and personal communication). In each case, a single protein is missing except after infection with *sus* mutants in gene 10 where protein NP3 is synthesized in small amounts due to a polar effect [8] or with *sus* mutants in gene 12 where the three related proteins P-NP1 (precursor), NP1 (appendage protein) and p12 (fragment) are lacking. The polar effect in the first case is indicated from the fact that *sus* mutants in cistron 10 complement poorly with either *sus* or *ts* mutants in cistron 11, coding for protein NP3, whereas *ts* mutants in cistron 10 complement well with mutants in cistron 11 [8].

Infection with mutant *sus*1(629) does not produce the synthesis of two early proteins, d and e. One of these two proteins is probably the product of cistron 1; the absence of the other may be due either to a polar effect or to the presence of a second mutation.

Mutant *sus*8(679), as well as other *sus* mutants in this cistron, also lack two proteins: the major head protein (HP1) and the fiber protein (HP3) [6, 17]. The possibility that the absence of both proteins is due to a double mutation is unlikely since two different mutants in this cistron have been analyzed. Two other possibilities are: (a) existence of a polar effect of the *sus* mutation in cistron 8, coding for one of the two head proteins, towards a new cistron, as yet unidentified, coding for the other protein; (b) cistron 8 codes for a precursor protein which is cleaved to the two head proteins HP1 and HP3, as happens in other cases [32, 33].

The other phage structural proteins, except HP0 and HP2, have been assigned to specific genes (Fig. 13). It is interesting to note the clustering of these genes (8 to 12), as well as cistron 7 which plays an important role in head morphogenesis [29], in the middle of the genetic map, as happens in other phages [34 – 37]. All of these are late proteins. As Schachtele *et al.* [38] have shown, the late RNA is transcribed from the heavy DNA strand. From the polar effect of cistron 10 towards cistron 11 [8] and from the high-molecular-weight fragment produced after infection with mutant *sus*12(47) [14], as well as from its location in the genetic map [8], one can conclude that the transcription of these genes takes place from left to right. Thus, the transcription of cistrons 7 to 12 probably takes place from left to right from the heavy DNA strand.

The protein products of cistrons 1, 3, 6 and 17, involved in DNA synthesis, have been characterized as early proteins. The protein products of cistrons 2 and 5, also involved in DNA synthesis, and cistron 4, involved in late control, are probably also early proteins. Since Schachtele *et al.* [38] have shown that the transcription of the early genes takes place from the light strand, cistrons 1 to 6, as well as cistron 17, are probably transcribed from right to left from the light strand. Thus, there are probably at least two sites for the initiation of the transcription of early genes from right to left from the light strand, located to the right of genes 6 and 17. We do not know yet whether covalently closed DNA circles are formed after Φ29 infection; if that were the case, the genes involved in DNA synthesis would be closely linked as happens in phage T7 [39].

Cistrons 15 and 16 code for late proteins. It is presently unknown whether genes 13 and 14 code for early or late proteins, although if cistron 14 is involved in the final lysis of the bacteria it probably codes for a late protein. Thus, if genes 13 and 14 code for late proteins, all the transcription of the late genes would take place from left to right from the heavy DNA strand, starting at the left of cistron 7. By using two-dimensional electrophoresis [28] we are trying to determine

the protein products of these two cistrons to decide whether they are early or late proteins. Also, by using the *Eco*RI fragments of Φ29 DNA we are presently analyzing the transcription map of the phage DNA to locate the regions coding for early and late RNA, respectively.

The excellent technical assistance of J. de la Torre is gratefully acknowledged. This investigation has been aided by Grants from *Comisión Asesora para el Desarrollo de la Investigación Científica y Técnica, Comisión Administradora del Descuento Complementario (I.N.P.)* and *Dirección General de Sanidad.* J.L.C., A.C., F.J. and R.P.M. are fellows of *Fondo Nacional para la Formación de Personal Investigador.*

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